

Influence of nitrate and nitrite on thyroid hormone responsive and stress-associated gene expression in cultured *Rana catesbeiana* tadpole tail fin tissue

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Nitrate and nitrite are common aqueous pollutants that are known to disrupt the thyroid axis. In amphibians, thyroid hormone (TH)-dependent metamorphosis is affected, although whether the effect is acceleration or deceleration of this developmental process varies from study to study. One mechanism of action of these nitrogenous compounds is through alteration of TH synthesis. However, direct target tissue effects on TH signaling are hypothesized. The present study uses the recently developed cultured tail fin biopsy (C-fin) assay to study possible direct tissue effects of nitrate and nitrite. Tail biopsies obtained from premetamorphic *Rana catesbeiana* tadpoles were exposed to 5 and 50 mg/L nitrate (NO₃-N) and 0.5 and 5 mg/L nitrite (NO₂-N) in the absence and presence of 10 nM T₃. Thyroid hormone receptor β (TR β) and Rana larval keratin type I (RLKI), both of which are TH-responsive gene transcripts, were measured using quantitative real time polymerase chain reaction. To assess cellular stress which could affect TH signaling and metamorphosis, heat shock protein 30, and catalase (CAT) transcript levels were also measured. We found that nitrate and nitrite did not significantly change the level of any of the four transcripts tested. However, nitrate exposure significantly increased the heteroscedasticity in response of TR β and RLKI transcripts to T₃. Alteration in population variation in such a way could contribute to the previously observed alterations of metamorphosis in frog tadpoles, but may not represent a major mechanism of action.

Keywords: nitrate, nitrite, frog, thyroid hormone, metamorphosis, C-fin, organ culture assay, quantitative real time polymerase chain reaction

INTRODUCTION

Currently over 100,000 manufactured chemicals are produced in the marketplace (European Union Commission, 2006). Many of these chemicals have endocrine disrupting abilities and more specifically, are disruptors of the thyroid axis. Most endocrine disruptors can be classified as plasticizers, pesticides, industrial chemicals, heavy metals, or plant and fungal compounds; however, ions such as nitrate (NO₃⁻) and nitrite (NO₂⁻) have endocrine disrupting abilities as well (Crain, 2000; Sampat, 2000; Gray et al., 2001). Environmental nitrate can come from many sources including agricultural fertilizer, waste from animal production, and burning fossil fuels, industrial effluent, and wastewater treatment plant discharges (Rouse et al., 1999; Camargo et al., 2004; De Groef et al., 2006).

In the US, the current public health maximal level for safe drinking water is 10 mg/L nitrate (measured as NO₃-N) and 1 mg/L nitrite (NO₂-N; US EPA, 2006, 2009). In Canada, the Canadian Council of Ministers of the Environment (CCME) guideline for the protection of aquatic life has set the maximum level of nitrate at 13 mg/L (NO₃⁻/L) in freshwater and 16 mg/L (NO₃⁻/L) in marine water; the level for nitrite in freshwater is 60 μ g/L (NO₂⁻/L) and there is no level set for marine water (CCME, 2007). Health

Canada has set the maximal allowable concentration in drinking water at 10 mg/L nitrate (NO₃-N) and 3.2 mg/L nitrite (NO₂-N; Health Canada, 2008). Nitrate concentrations have been found as high as 25 mg/L NO₃-N in surface waters and 100 mg/L NO₃-N in ground water, yet there is currently no guideline for the protection of wildlife (Rouse et al., 1999; Camargo et al., 2004).

In aquatic environments, nitrogen exists in four forms in descending order of toxicity: ammonium ion, ammonia, nitrite, and nitrate. Although nitrate is the least toxic form of the four, it is the most stable and therefore the most abundant. Under aerobic conditions, ammonia and ammonium can be oxidized to nitrite by *Nitrosomonas* bacteria, and then to nitrate by *Nitrobacter* and *Nitrospira* bacteria (Sharma and Ahlert, 1977). When oxygen is low, denitrifying bacteria can use nitrate as a terminal electron acceptor and make nitrogen gas (N₂; reviewed in Camargo et al., 2005).

Aquatic animals are exposed to nitrate and nitrite through ingestion or epithelial absorption across skin or gills (Onken et al., 2003). High levels of these contaminants cause methemoglobinemia, also called “brown blood” disease in fish and amphibians and “blue baby” syndrome in humans. Methemoglobin is formed from nitrate/nitrite-induced oxidation of hemoglobin, which prevents

normal oxygen binding and leads to hypoxia (Porter et al., 1999). Toxicity of nitrite and nitrate depends on body size and developmental stage, increases with increasing concentration and exposure time, and decreases with water salinity and environmental adaptation (Rouse et al., 1999; Camargo et al., 2004).

In addition to the toxic effects of nitrate and nitrite, exposure to these chemicals adversely affects the thyroid axis in multiple vertebrate species (reviewed in Edwards et al., 2006). For example, high doses of nitrate caused goiter and depressed serum thyroxine (T_4) and 3,5,3'-triiodothyronine (T_3) in rats and sheep (Zaki et al., 2004) and nitrite decreased serum T_4 while T_3 levels were unchanged in the sea bream (Deane et al., 2007). Toad and frog tadpoles exposed to nitrate exhibit altered metamorphic development; a TH-dependent process (Wyngaarden et al., 1952, 1953; Xu and Oldham, 1997; Edwards et al., 2006; Ortiz-Santaliestra and Sparling, 2007). Although these observations can be explained, in part, by competition of nitrate and nitrite with iodine uptake, transport, and retention in the thyroid gland that impairs TH synthesis (Crow et al., 2001; Hampel and Zollner, 2004), the contribution of nitrate and nitrite to alteration of TH signaling pathways at the cellular level in amphibian target tissues is not known.

The present study uses the recently developed "C-fin" assay to expose *Rana catesbeiana* premetamorphic tadpole tail fin biopsies to nitrate and nitrite with or without T_3 to determine if nitrate and nitrite affect TH-signaling within a TH-responsive tissue directly. We assessed TH-signaling by quantifying the levels of TH-responsive gene transcripts, thyroid hormone receptor β (*TR β*) and *Rana* larval type I keratin (*RLKI*), as well as cellular stress markers, heat shock protein (*HSP30*), and catalase (*CAT*). Alteration of the transcriptome is an essential component in TH-mediated tadpole metamorphosis (Shi, 2000) and part of the change in the tail transcriptome includes an increase in *TR β* transcripts and a decrease in *RLKI* transcripts (Domanski and Helbing, 2007). There is considerable precedent linking *TR β* transcript levels to progression through TH-dependent metamorphosis where perturbations from expected levels are indicative of altered postembryonic development (Crump et al., 2002; Opitz et al., 2006; Veldhoen et al., 2006a; Zhang et al., 2006; Helbing et al., 2007a,b; Ji et al., 2007; Skirrow et al., 2008).

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Premetamorphic *R. catesbeiana* tadpoles were caught locally (Victoria, BC, Canada) or purchased from Ward's Natural Science Ltd. (St. Catharines, ON, Canada). Taylor and Kollros (TK; Taylor and Kollros, 1946) stage VI–VIII animals were used. Animals were housed in the University of Victoria aquatics facility and maintained in 100 gallon fiberglass tanks containing recirculating water at 12°C with exposure to natural daylight. Tadpoles were fed daily with spirulina (Aquatic ELO-Systems, Inc., FL, USA). Animals used in this study were treated and maintained in accordance with the guidelines of the Canadian Council on Animal Care.

ORGAN CULTURE OF TAIL FIN BIOPSIES

Preparation of the tail fin biopsy cultures was adapted from conditions described previously (Veldhoen et al., 2006b; Ji et al.,

2007). Premetamorphic (TK stage VI–VIII; Taylor and Kollros, 1946) *R. catesbeiana* tadpoles were euthanized in 0.1% tricaine methanesulfonate (Syndel Laboratories, Vancouver, BC, Canada) in 25 mM sodium bicarbonate, and subsequently washed four times in 125 mL per tadpole of sterile magnesium-free (MFM) solution (7.5 mM Tris–HCl pH 7.6, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO_3 , 0.88 mM CaCl_2). Eight biopsies were obtained per animal ($n = 7$ –16 animals), from the dorsal and ventral tail fins using a 6 mm dermal biopsy punch (Miltex, Inc., York, PA, USA), to allow the assessment of eight treatments per animal. Sixteen animals were used for each exposure.

Test chemicals were prepared in water as 1,000 \times concentrates and stored at -20°C . They were applied in equal volumes at 1 μL chemical stock/mL of media. Treatments included: a vehicle control (NaOH), sodium gluconate control (Na-G, used as a control for sodium; CAS S-2054, Sigma-Aldrich), sodium nitrate (NaNO_3 ; CAS BP360-500g, Fisher; measured as 5 and 50 mg/L $\text{NO}_3\text{-N}$), or sodium nitrite (NaNO_2 ; CAS S2252-500g, >99.5% purity, Sigma-Aldrich; measured as 0.5 and 5 mg/L $\text{NO}_2\text{-N}$), in the absence and presence of 10 nM T_3 (prepared as a 10^{-5} M stock in 400 μM NaOH), as well as a 10 nM T_3 treatment alone. Where treatments did not include T_3 , an equal volume of NaOH vehicle was applied to a final concentration of 400 nM. This concentration did not affect the medium pH. Biopsies were cultured individually in 1 mL 70% strength Leibovitz's L15 medium (Gibco, Invitrogen) supplemented with 10 mM HEPES pH 7.5, 50 units/mL penicillin G sodium, 50 $\mu\text{g/mL}$ streptomycin sulfate (Gibco, Invitrogen), and 50 $\mu\text{g/mL}$ neomycin (Sigma-Aldrich), using 24-well culture plates (Primaria, BD Biosciences) at 25°C in air for 48 h.

The biopsies were pretreated with 0.5 mL of the appropriate concentration of the test chemical or NaOH control in culture media for 2 h prior to the addition of T_3 . After the 2 h incubation, 0.5 mL of the appropriate concentration of the test chemical plus 20 nM T_3 (in 800 μM NaOH) were added into the wells giving a final concentration of 10 nM T_3 (in 400 nM NaOH). For the wells not containing T_3 , 0.5 mL of the appropriate concentration of the test chemical plus 800 μM NaOH (for a final concentration of 400 nM NaOH) were added. At the end of the 48 h incubation period for each treatment, the biopsy was stored in 100 μL of RNeasy lysis buffer (Qiagen, Crawley, UK) for 24 h at 4°C and then transferred to -20°C until it was processed for RNA.

ISOLATION OF RNA AND QUANTIFICATION OF GENE EXPRESSION

RNA was isolated using TRIzol reagent as described previously (Hinther et al., 2010a,b). cDNA was synthesized from 5 μL ($\sim 0.5 \mu\text{g}$) total RNA as per manufacturer's protocol using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) as described in (Hinther et al., 2010b). The cDNA products were diluted fivefold prior to PCR amplification and stored at -20°C .

The levels of mRNAs encoding *TR β* , *RLKI*, *HSP30*, *CAT*, and ribosomal protein L8 (*rpl8*) were determined using a MX3005P real time quantitative PCR system (Stratagene, La Jolla, CA, USA) using gene-specific primers as described previously (Hinther et al., 2010b). Expression profiles of the *rpl8* transcript normalizer were invariant ($p = 0.998$ and 0.950 , nitrate and nitrite data sets, respectively). The amplified DNA signals for all QPCR reactions were

evaluated for specificity based upon their thermodenaturation profiles. Data that failed to produce a profile indicative of gene target-specific detection were removed before analysis. If a control condition did not pass the quality measure above for a given animal, then the data for all conditions associated with that animal were removed for that gene transcript due to the repeated measures nature of the data set.

STATISTICAL ANALYSES

Statistical analyses were performed using PASW 18.0 (Chicago, IL, USA) software. The C-fin data were not normally distributed based upon the Shapiro–Wilk test. We used the Friedman and paired Wilcoxon tests since these data were generated from a repeated measures type of experimental design. Homogeneity of variance was determined using the Levene's test. Correlation coefficients were generated using Spearman's rho.

The data were analyzed in two ways: First, the test chemical results in the absence of T_3 were examined relative to the vehicle control. Second, the test chemicals in combination with T_3 results were compared relative to T_3 alone. In the latter case, the response to a test chemical in the presence of T_3 was expressed as a fold change relative to the response to T_3 alone for each individual. This approach reduces the effect of inter-animal variation, enabling us to better identify chemical-induced perturbations relative to each individual's ability to respond to T_3 . Therefore the T_3 values in this comparison were given a value of one and the graphs show the fold change relative to the T_3 -induced response. Statistical significance was identified when $p < 0.05$.

RESULTS AND DISCUSSION

In order to validate the assay, we first examined the biopsy responses to T_3 treatment alone. **Figure 1A** shows the biological variation of the controls and the relative variation in T_3 response before taking repeated measures into account. **Figure 1B** shows the data after normalizing the data to the individual animal's baseline transcript levels into such that every control animal was assigned a value of 1. T_3 treatment alone increased the *TRβ* transcript levels by a median 7.7-fold ($p = 0.0001$, Wilcoxon, $n = 23$; **Figure 1**), decreased *RLKI* transcript levels by 2.9-fold ($p = 0.0001$, Wilcoxon, $n = 26$; **Figure 1**), increased *HSP30* transcript levels by 1.7-fold ($p = 0.024$, Wilcoxon, $n = 31$; **Figure 1**), and reduced *CAT* transcript levels by a median 1.3-fold ($p = 0.024$, Wilcoxon, $n = 32$; **Figure 1**). All transcript responses were similar to previous observations (Hinther et al., 2010a,b, 2011).

The C-fin experimental design allows for the determination of relationship between the individual animal's baseline transcript levels and the extent of change in transcript level in response to chemical treatment. We examined the correlation between the baseline (control) levels of each transcript to each other and to the individual's level of fold induction in response to T_3 exposure (**Table 1**). A strong negative correlation between baseline transcript levels and the degree of fold response to T_3 for all four transcripts was observed (**Table 1**). This observation was consistent with previously reported observations for *TRβ* and *RLKI* transcripts (Hinther et al., 2010a). A strong negative correlation was observed between the baseline levels of *CAT* and the

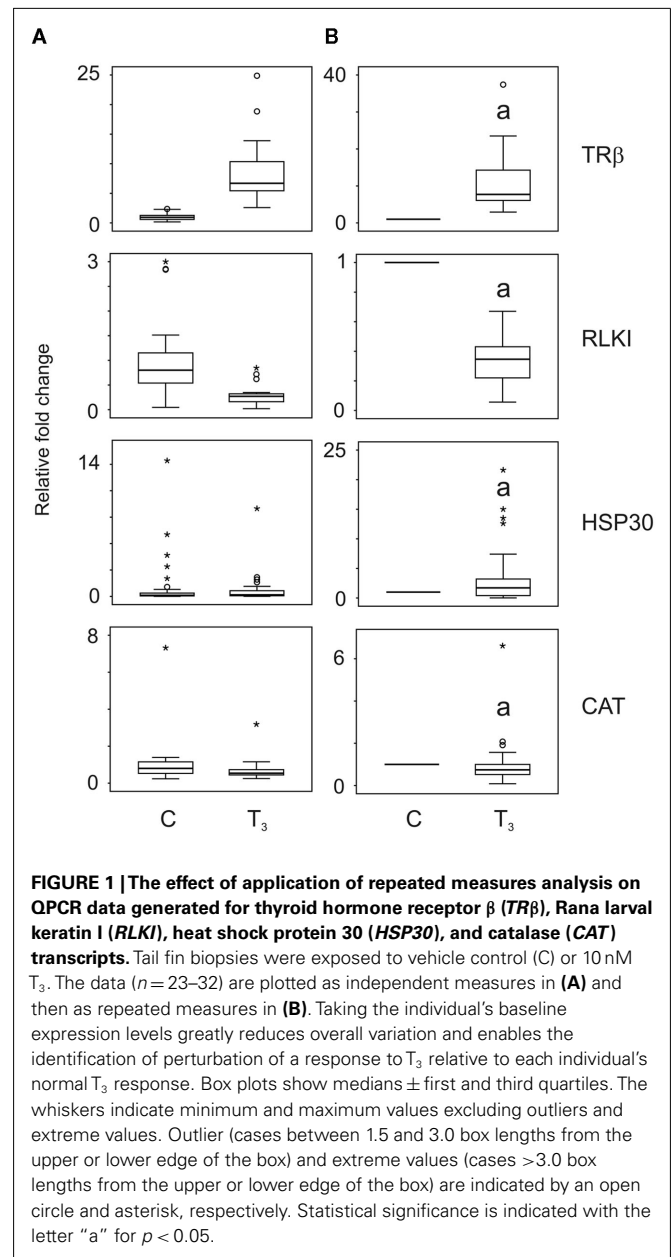


FIGURE 1 | The effect of application of repeated measures analysis on QPCR data generated for thyroid hormone receptor β (*TRβ*), Rana larval keratin I (*RLKI*), heat shock protein 30 (*HSP30*), and catalase (*CAT*) transcripts. Tail fin biopsies were exposed to vehicle control (C) or 10 nM T_3 . The data ($n = 23$ – 32) are plotted as independent measures in (A) and then as repeated measures in (B). Taking the individual's baseline expression levels greatly reduces overall variation and enables the identification of perturbation of a response to T_3 relative to each individual's normal T_3 response. Box plots show medians \pm first and third quartiles. The whiskers indicate minimum and maximum values excluding outliers and extreme values. Outlier (cases between 1.5 and 3.0 box lengths from the upper or lower edge of the box) and extreme values (cases >3.0 box lengths from the upper or lower edge of the box) are indicated by an open circle and asterisk, respectively. Statistical significance is indicated with the letter "a" for $p < 0.05$.

T_3 -dependent reduction of *RLKI* transcripts and *RLKI* and the T_3 -dependent decrease of the *CAT* transcript (**Table 1**). A positive correlation was observed between the baseline levels of *HSP30* and the T_3 -dependent increase of *TRβ* mRNAs (**Table 1**).

Exposure to 0.5 and 5 mg/L $\text{NO}_2\text{-N}$ (in the form of NaNO_2) for 48 h did not have any effect on the TH-responsive gene transcripts, *TRβ* and *RLKI*, in the absence ($p = 0.683$ and 0.257 , respectively; Friedman) or presence of T_3 ($p = 0.282$ and 0.751 , respectively; Friedman; **Figure 2**). Exposure to 5 mg/L sodium gluconate (Na-G; as a control for sodium) also did not result in a significant effect compared to the control ($p = 0.300$ – 0.875 , Wilcoxon; **Figure 2**).

Exposure to 5 and 50 mg/L $\text{NO}_3\text{-N}$ (in the form of NaNO_3) did not result in a change in *TRβ* and *RLKI* transcript levels

Table 1 | Spearman's rho correlation analysis comparing baseline transcript levels with extent of (fold) induction in response to T_3 treatment.

			Fold induction by T_3			
			TR β	RLKI	HSP30	CAT
Baseline	TR β	Correlation coefficient	−0.645	−0.065	0.159	0.018
		<i>p</i> Value	0.000*	0.396	0.240	0.468
		<i>N</i>	23	19	22	23
	RLKI	Correlation coefficient	0.058	−0.570	−0.124	−0.350
		<i>p</i> Value	0.407	0.001*	0.278	0.040*
		<i>N</i>	19	26	25	26
	HSP30	Correlation coefficient	0.452	−0.015	−0.552	0.025
		<i>p</i> Value	0.017*	0.471	0.001*	0.448
		<i>N</i>	22	25	31	31
	CAT	Correlation coefficient	0.136	−0.465	−0.006	−0.618
		<i>p</i> Value	0.267	0.008*	0.487	0.000*
		<i>N</i>	23	26	31	32

Significance is indicated with an asterisk.

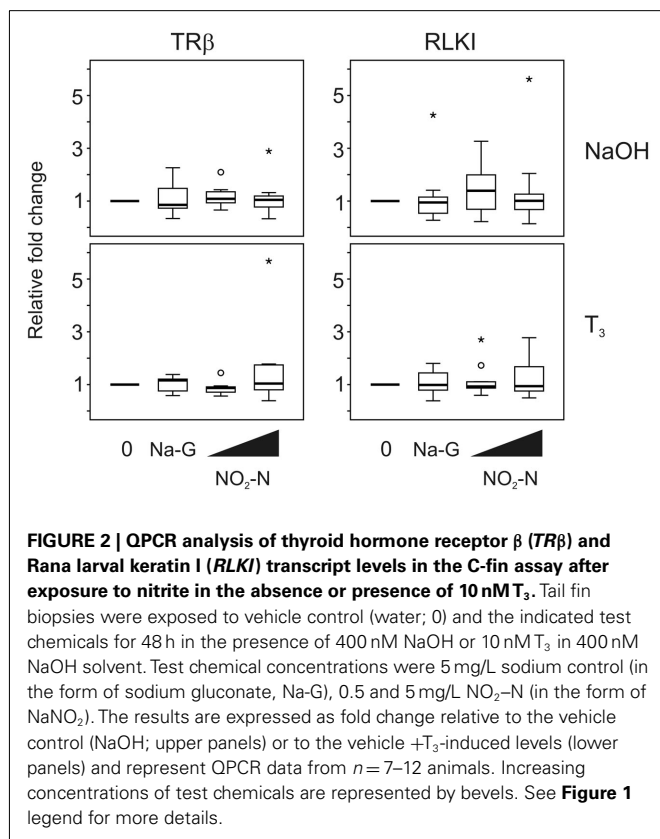


FIGURE 2 | QPCR analysis of thyroid hormone receptor β (TR β) and Rana larval keratin I (RLKI) transcript levels in the C-fin assay after exposure to nitrite in the absence or presence of 10 nM T_3 . Tail fin biopsies were exposed to vehicle control (water; 0) and the indicated test chemicals for 48 h in the presence of 400 nM NaOH or 10 nM T_3 in 400 nM NaOH solvent. Test chemical concentrations were 5 mg/L sodium control (in the form of sodium gluconate, Na-G), 0.5 and 5 mg/L NO_2 -N (in the form of $NaNO_2$). The results are expressed as fold change relative to the vehicle control (NaOH; upper panels) or to the vehicle + T_3 -induced levels (lower panels) and represent QPCR data from $n = 7$ –12 animals. Increasing concentrations of test chemicals are represented by bevels. See Figure 1 legend for more details.

in the absence ($p = 0.565$ and 0.913 , respectively, Friedman; Figure 3) or presence of T_3 ($p = 0.066$ and 0.529 , respectively,

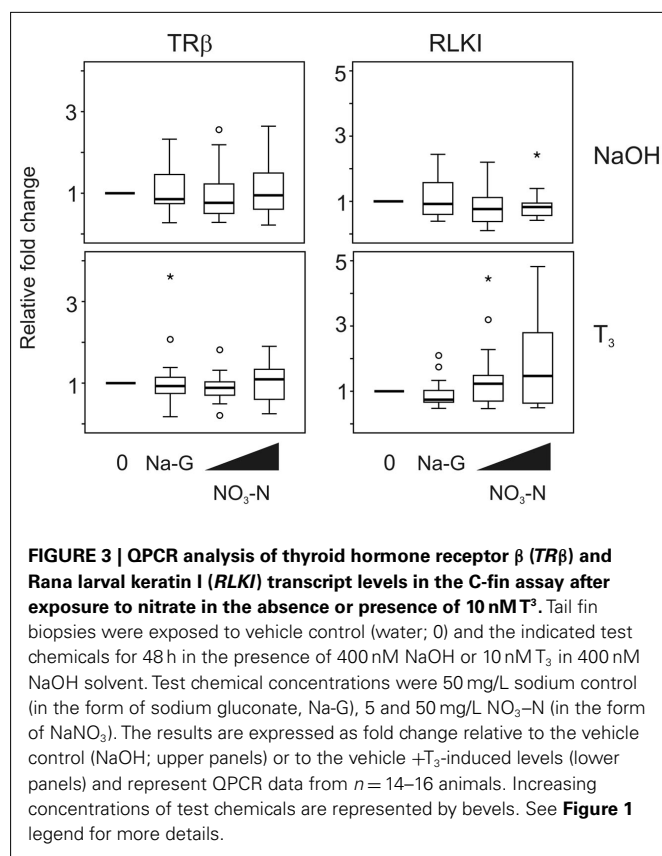


FIGURE 3 | QPCR analysis of thyroid hormone receptor β (TR β) and Rana larval keratin I (RLKI) transcript levels in the C-fin assay after exposure to nitrate in the absence or presence of 10 nM T_3 . Tail fin biopsies were exposed to vehicle control (water; 0) and the indicated test chemicals for 48 h in the presence of 400 nM NaOH or 10 nM T_3 in 400 nM NaOH solvent. Test chemical concentrations were 50 mg/L sodium control (in the form of sodium gluconate, Na-G), 5 and 50 mg/L NO_3 -N (in the form of $NaNO_3$). The results are expressed as fold change relative to the vehicle control (NaOH; upper panels) or to the vehicle + T_3 -induced levels (lower panels) and represent QPCR data from $n = 14$ –16 animals. Increasing concentrations of test chemicals are represented by bevels. See Figure 1 legend for more details.

Friedman; Figure 3). The 50 mg/L sodium control (in the form of sodium gluconate, Na-G) in this experiment had no effect as well ($p = 0.480$ – 1.000 , Wilcoxon; Figure 3).

Exposure to 0.5 and 5 mg/L NO_2 -N did not affect HSP30 and CAT transcript levels in the absence ($p = 0.444$ and 0.185 , respectively, Friedman; Figure 4) or presence of T_3 ($p = 0.570$ and 0.779 , respectively, Friedman; Figure 4). Exposure to 5 mg/L Na-G also did not result in a significant effect ($p = 0.438$ – 0.717 , Wilcoxon; Figure 4).

Exposure to 5 and 50 mg/L NO_3 -N did not result in a change in stress-responsive transcript levels in the absence ($p = 0.282$ and 0.819 , HSP30 and CAT transcripts respectively, Friedman) or presence of T_3 ($p = 0.074$ and 0.819 , respectively, Friedman; Figure 5). Exposure to 50 mg/L Na-G had no effect on the stress-responsive transcripts ($p = 0.796$ – 1.000 , Wilcoxon; Figure 5).

Changes in population variation have been associated with endocrine disruptive events and exposure to pollutants (Orlando and Guillette, 2001). An increase in variance is often found in contaminant-exposed sites compared with reference site populations, in part, due to varying individual responses to the environmental stressor. Since more individuals are at the perimeter of a population range away from the more homogeneous central part of the range, the contaminant-exposed population is less able to adapt to environmental stress and may require additional energy budget expenditures to survive (Orlando and Guillette, 2001). Thus, variation can represent an additional indicator of population health not necessarily captured by measures of central

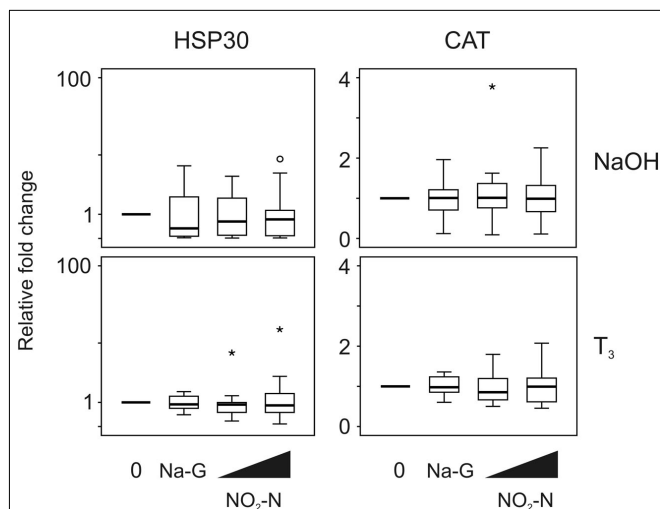


FIGURE 4 | QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to nitrite in the absence or presence of 10 nMT₃. Tail fin biopsies were exposed to vehicle control (water; 0) and the indicated test chemicals for 48 h in the presence of 400 nM NaOH or 10 nMT₃ in 400 nM NaOH solvent. Test chemical concentrations were 5 mg/L sodium control (in the form of sodium gluconate, Na-G), 0.5 and 5 mg/L NO₂-N (in the form of NaNO₂). The results are expressed as fold change relative to the vehicle control (NaOH; upper panels) or to the vehicle +T₃-induced levels (lower panels) and represent QPCR data from *n* = 15–16 animals. Increasing concentrations of test chemicals are represented by bevels. See **Figure 1** legend for more details.

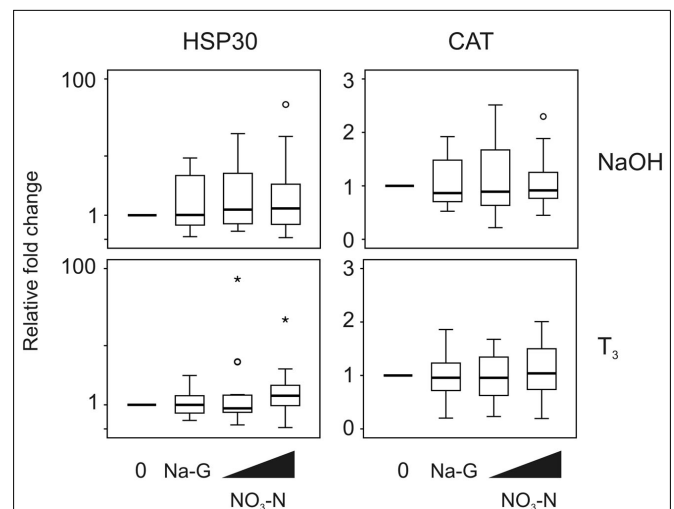


FIGURE 5 | QPCR analysis of heat shock protein 30 (*HSP30*) and catalase (*CAT*) transcript levels in the C-fin assay after exposure to nitrate in the absence or presence of 10 nMT₃. Tail fin biopsies were exposed to vehicle control (water; 0) and the indicated test chemicals for 48 h in the presence of 400 nM NaOH or 10 nMT₃ in 400 nM NaOH solvent. Test chemical concentrations were 50 mg/L sodium control (in the form of sodium gluconate, Na-G), 5 and 50 mg/L NO₃-N (in the form of NaNO₃). The results are expressed as fold change relative to the vehicle control (NaOH; upper panels) or to the vehicle +T₃-induced levels (lower panels) and represent QPCR data from *n* = 16 animals. Increasing concentrations of test chemicals are represented by bevels. See **Figure 1** legend for more details.

tendency (Orlando and Guillelte, 2001). No alterations in heteroscedasticity were observed for any transcripts between the Na-G controls or the nitrite treatments (**Table 2**). This was also the case for nitrate in the absence of hormone. However, when T₃ was present, nitrate exposure affected the degree of heteroscedasticity in both *TRβ* and *RLKI* mRNAs, but not *HSP30* or *CAT* transcripts (**Table 2**). A change in heteroscedasticity, as observed with nitrate exposure, suggests an alteration in the response to TH at the tissue level that is consistent with the conflicting acceleratory and inhibitory effects on TH-dependent processes that have previously been observed (Xu and Oldham, 1997; Edwards et al., 2006; Ortiz-Santaliestra and Sparling, 2007). The data in the present study suggest that nitrate and nitrite differ in cellular effects on TH signaling while not eliciting stress responses in the TH-responsive tail fin tissue. Moreover, direct cellular effects of nitrate on peripheral tissues as a mechanism in influencing metamorphosis still remains a possibility but that this effect is not straightforward. Examination of additional time points would be useful to evaluate whether TH-mediated response kinetics are altered.

It has been postulated that nitrite and nitrate could act as nitric oxide donors through a non-genomic mechanism (Guillelte and Edwards, 2005; Hannas et al., 2010). Nitric oxide donors have been shown to mimic the ability of T₄ to suppress catalase enzyme activity associated with tail shortening and apoptosis *in vitro* (Kashiwagi et al., 1999). However, a definitive connection between nitrate and nitrite and nitric oxide production in

Table 2 | Analysis of variation using Levine's test.

Treatment	Transcript	Levene statistic	<i>p</i> Value
NO ₂ -N	<i>TRβ</i>	0.484	0.624
	<i>RLKI</i>	0.261	0.772
	<i>HSP30</i>	0.563	0.574
	<i>CAT</i>	0.526	0.595
NO ₃ -N	<i>TRβ</i>	0.183	0.834
	<i>RLKI</i>	1.073	0.352
	<i>HSP30</i>	2.891	0.066
	<i>CAT</i>	0.348	0.708
NO ₂ -N + T ₃	<i>TRβ</i>	2.786	0.088
	<i>RLKI</i>	0.174	0.841
	<i>HSP30</i>	1.698	0.196
	<i>CAT</i>	0.205	0.815
NO ₃ -N + T ₃	<i>TRβ</i>	3.449	0.041*
	<i>RLKI</i>	7.542	0.002*
	<i>HSP30</i>	1.254	0.296
	<i>CAT</i>	0.102	0.903

Groups with significant heteroscedasticity are indicated with an asterisk.

amphibian tissues has not been established, although this relationship has been shown in *Daphnia* (Hannas et al., 2010). The fact that nitrate elicited some response whereas nitrite did not on cultured tail fin suggest that this influence could be limited. Direct effects of nitrate and nitrite upon other amphibian tissues (such as the thyroid gland) through genomic and/or non-genomic

methods from tissue culture experiments and comparison to molecular responses elicited from whole animal exposures remain to be determined.

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